Supplementary Information

Quantitative proteomic analysis of signalosome dynamics in primary T cells identifies the CD6 surface receptor as a Lat-independent TCR signaling hub

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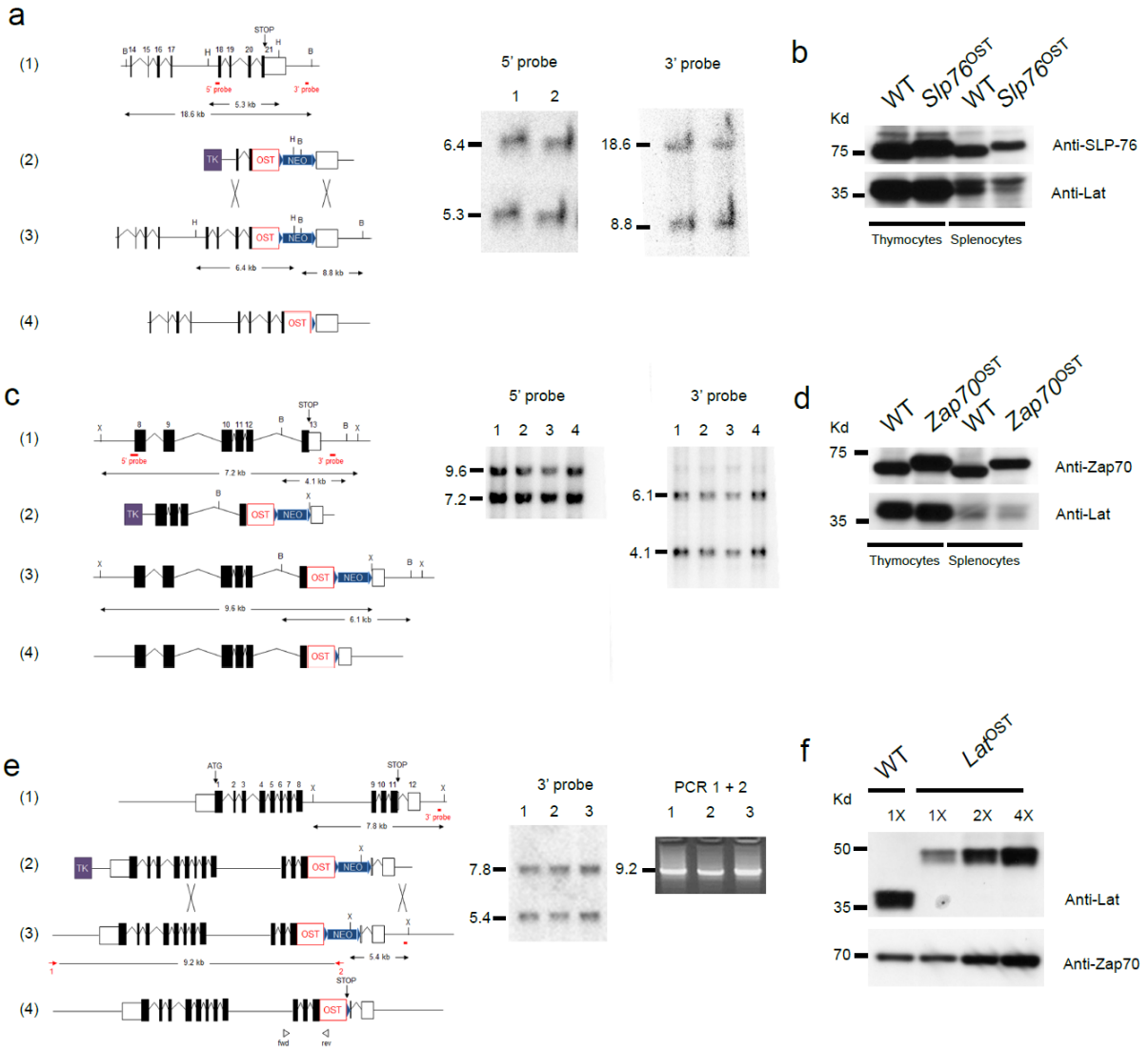
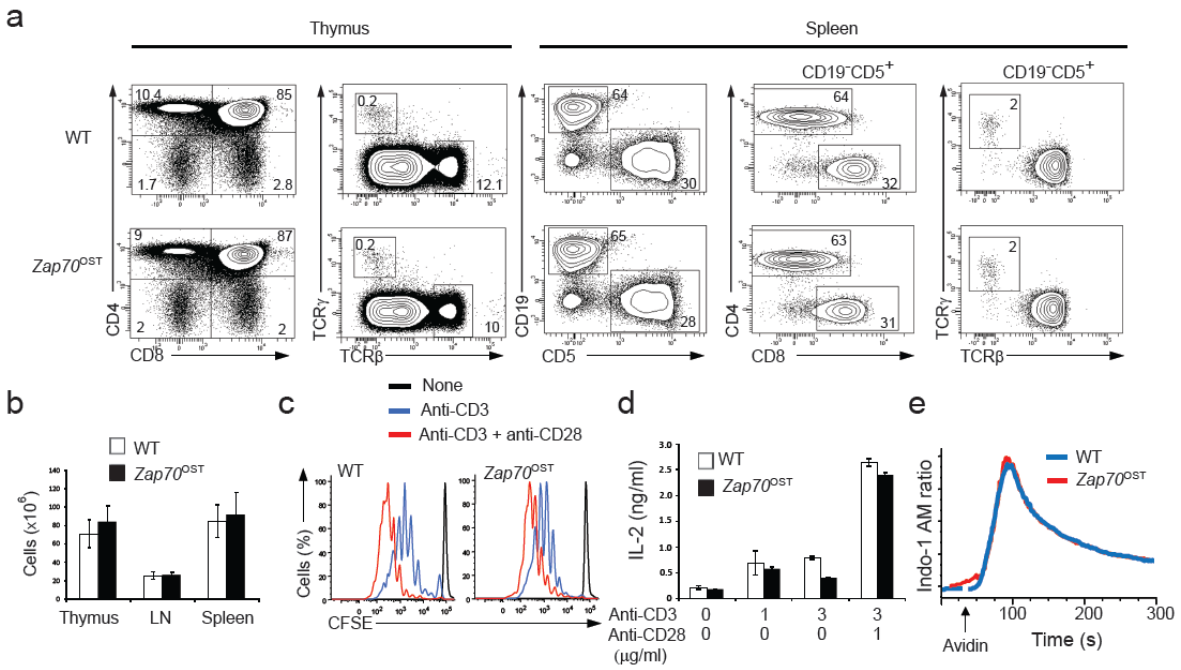
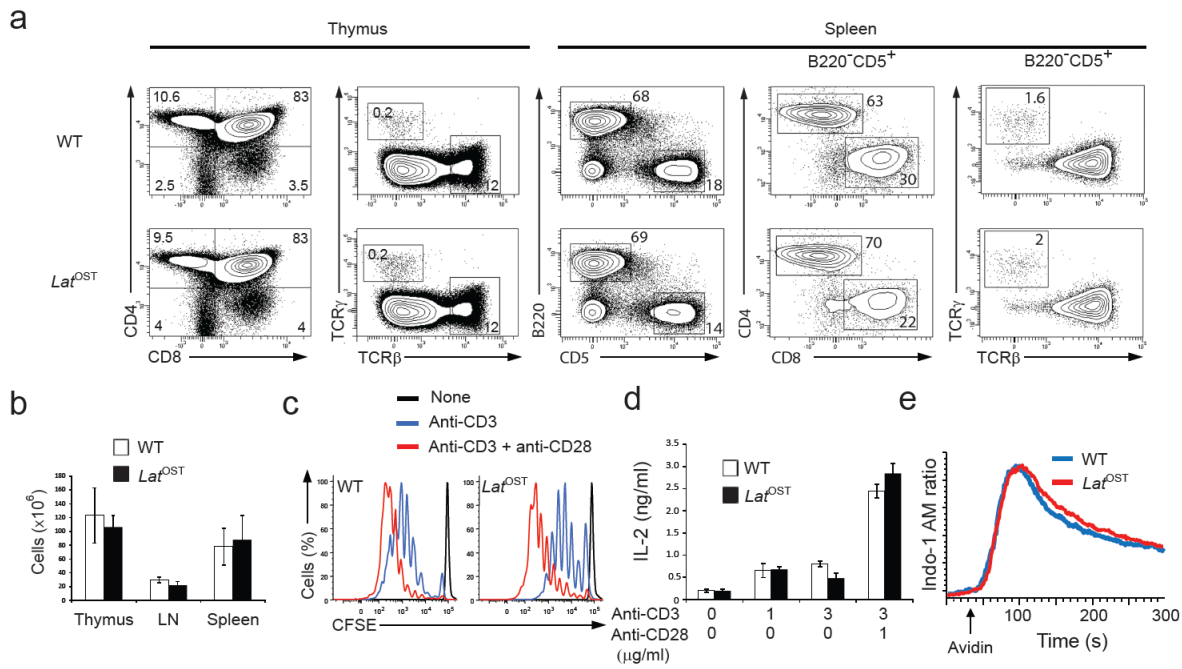


Supplementary Figure 1. Generation and validation of knock-in mice expressing endogenous SLP-76, Zap70 and Lat proteins tagged with a One-STrEP-tag (OST) sequence. (**a**) Strategy used to produce *Slp76*^{OST} knock-in mice. Left panel: (1) Partial restriction map of the *Slp76* (*Lcp2*) gene. Exons are shown as filled black boxes and numbered. The stop codon (STOP) is indicated. The 5′- and 3′-single copy probes used to verify proper homologous recombination events by Southern blot analysis are shown in red. B: *BamHI*, H: *HindIII*. (2) Targeting vector used for the introduction of the OST sequence in the *Slp76* gene. A OST-(Stop)$_2$-*lox*P-tACE-CRE-PGK-gb2-*neo*^{r}-*lox*P cassette (OST-NEO) was introduced at the 3′ end of the *Slp76* coding sequence (see Online Methods). The tACE-CRE-PGK-gb2-*neo*^{r} sequence is bracketed by *lox*P sites (triangles) and directs its own excision as it passes through the male germline. TK: thymidine kinase expression cassette. (3) Structure of the targeted *Slp76*^{OST} allele following homologous recombination. (4) Structure of the *Slp76*^{OST} allele following homologous recombination and Cre-mediated self-excision of the tACE-CRE-PGK-gb2-*neo*^{r} cassette. Right panel: Southern blot analysis of two appropriately recombined ES clones. ES cell DNA was digested with

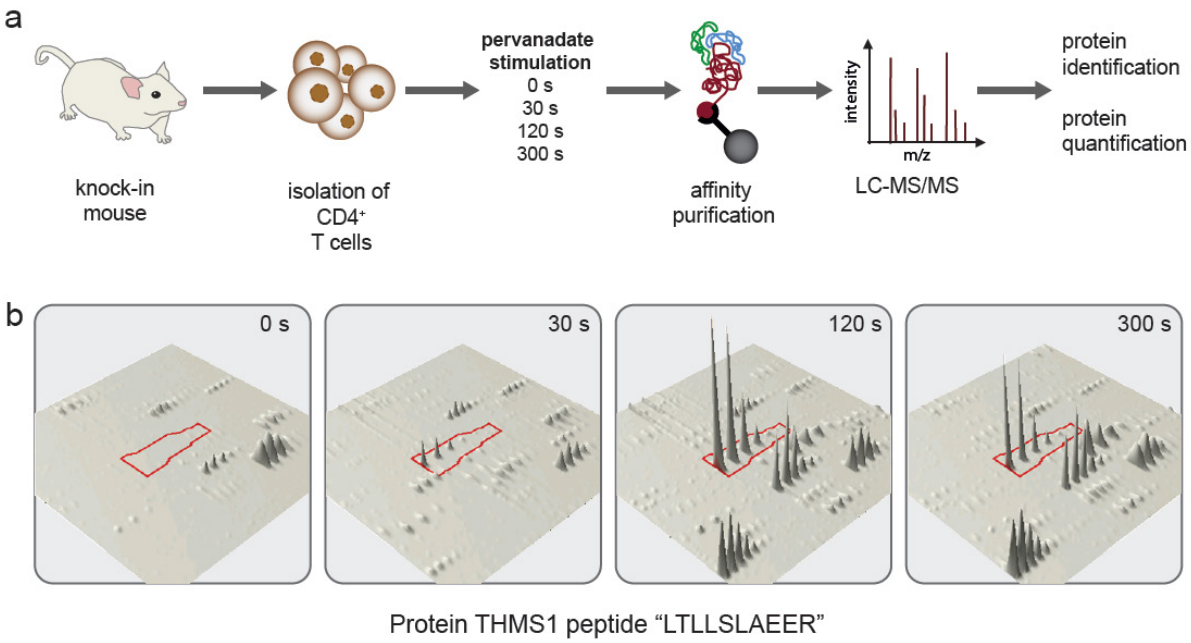
HindIII (5′ probe) or *BamHI* (3′ probe) and hybridized to the 5′ or 3′ single-copy probe shown in line 1 of the left panel. (**b**) Expression of the SLP-76 and SLP7-6-OST proteins in thymocytes and splenocytes from wild-type (WT) and *Slp76*^{OST} mice was detected using an anti-SLP-76. Blotting with an antibody against Lat served as a loading control. (**c**) Strategy used to produce *Zap70*^{OST} knock-in mice. Left panel: (1) Partial restriction map of the *Zap70* gene. Exons are shown as filled black boxes and numbered. The stop codon (STOP) is indicated. The 5′- and 3′-single copy probes used to verify proper homologous recombination events by Southern blot analysis are shown in red. B: *BamHI*, X: *XbaI*. (2) Targeting vector used for the introduction of the OST in the *Zap70* gene as described in (**a**). B: *BamHI*, X: *XbaI*. (3) Structure of the targeted *Zap70* ^{OST} allele following homologous recombination. (4) Structure of the *Zap70*^{OST} allele following homologous recombination and Cre-mediated self-excision of the tACE-CRE-PGK-gb2-*neo*^{r} cassette. Right panel: Southern blot analysis of four appropriately recombined ES clones. ES cell DNA was digested by *Xba*I (5′ probe) or *BamHI* (3′ probe) and hybridized to the 5′ or 3′single-copy probe shown in line 1 of the left panel. (**d**) Expression of Zap70 and Zap70-OST proteins in thymocytes and splenocytes from wild-type (WT) and *Zap70*^{OST} mice was detected using an anti-Zap70. Blotting with an antibody against Lat served as a loading control. (**e**) Strategy used to produce *Lat*^{OST} knock-in mice. Left panel: (1) Partial restriction map of the *Lat* gene. Exons are shown as filled black boxes and numbered. The initiation (ATG) and stop (STOP) codons are indicated. The 3′-single copy probe used to verify proper homologous recombination events by Southern blot analysis is shown in red. X: *XbaI*. (2) Targeting vector used for the introduction of the OST in the *Lat* gene as described in (**a**). X: *X*baI. (3) Structure of the targeted *Lat* ^{OST} allele following homologous recombination. The pair of PCR primers (red arrows) used to verify proper homologous recombination events at the 5′ end is shown (4) Structure of the *Lat* ^{OST} allele following homologous recombination and Cre-mediated self-excision of the tACE-CRE-PGK-gb2-*neo*^{r} cassette. Right panel: Southern blot analysis of three appropriately recombined ES clones. ES cell DNA was digested by *XbaI* and hybridized to the 5′ single-copy probe shown in line 1 of left panel. PCR analysis of tail DNA of the three ES clones using the pair of primers shown in line 4 of left panel. (**f**) Expression of Lat and Lat-OST proteins in thymocytes from wild-type (WT) and *Lat*^{OST} mice was detected using an anti-Lat. To estimate the levels reduction in Lat-OST as compared to Lat, increasing amounts (1X, 2X and 4X) of *Lat*^{OST} lysates were analyzed. Blotting with an antibody against Zap70 served as a loading control.



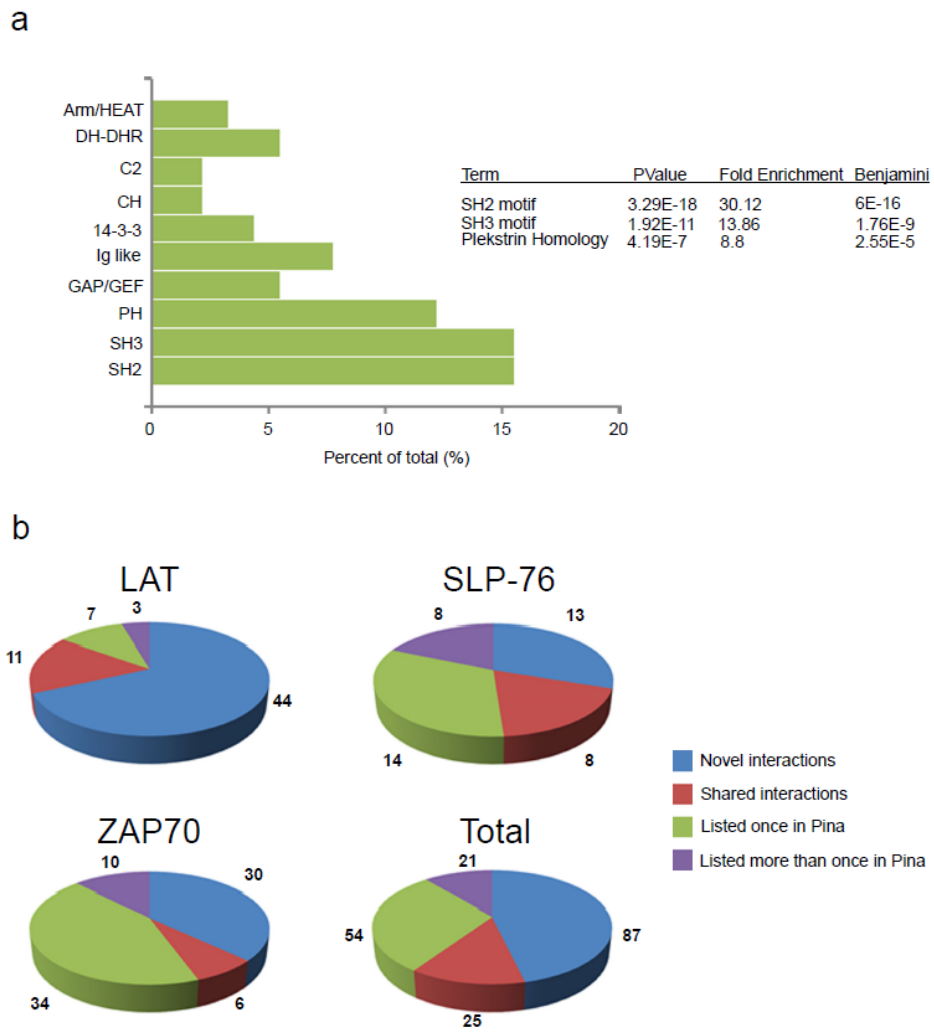
Supplementary Figure 2. Normal development and function of T cells isolated from knock-in mice homozygous for the *Zap70*^{OST} allele. (**a**) Flow cytometry analysis of thymus and spleen. Wild-type (WT) and *Zap70*^{OST} thymocytes were analyzed for expression of CD4 and CD8 (left) and TCRαβ and TCRγδ (right). Expression of CD4 and CD8 (left) and TCRαβ and TCRγδ (right) by wild-type (WT) and *Zap70*^{OST} thymocytes. Numbers adjacent to outlined areas indicate percent double-positive cells (top right), CD4$^+$ single-positive cells (top left), CD8$^+$ single-positive cells (bottom right) and double negative CD4$^-$CD8$^-$ cells (bottom left). Wild-type and *Zap70*^{OST} splenocytes were analyzed for expression of CD5 and CD19. Numbers adjacent to outlined areas indicate percent B cells (top left) and T cells (bottom right). Expression of CD4 and CD8 and of TCRαβ and TCRγδ by T cells (identified as CD19$^-$CD5$^+$ cells). Numbers adjacent to outlined areas indicate percent CD4$^+$ and CD8$^+$ cells (middle) and TCRαβ and TCRγδ cells (right). Data are representative of at least three experiments with two mice per genotype. (**b**) Cellularity of thymus, pooled axillary, brachial, inguinal and mesenteric lymph nodes (LN) and spleen of wild-type and *Zap70*^{OST} mice. Data are expressed as mean value ± SEM (n=6). (**c**) CFSE dilution by wild-type, and *Zap70*^{OST} CD4$^+$ T cells activated for 72 h with plate-bound anti-CD3 (3 μg/ml) in the presence or absence (key) of soluble anti-CD28 (1 μg/ml). Black lines, CFSE dilution at initiation of culture. (**d**) IL-2 in supernatants of wild-type, and *Zap70*^{OST} CD4$^+$ T cells activated for 72 h as in (**c**). (**e**) Changes in intracellular calcium in wild-type, and *Zap70*^{OST} CD4$^+$ T cells stimulated with biotinylated anti-CD3 and avidin. Arrow corresponds to the time of addition of avidin. Data are representative of at least two experiments. Wild-type and *Zap70*^{OST} CD8$^+$ T cells were analyzed in parallel and showed similar changes in intracellular calcium (data not shown).



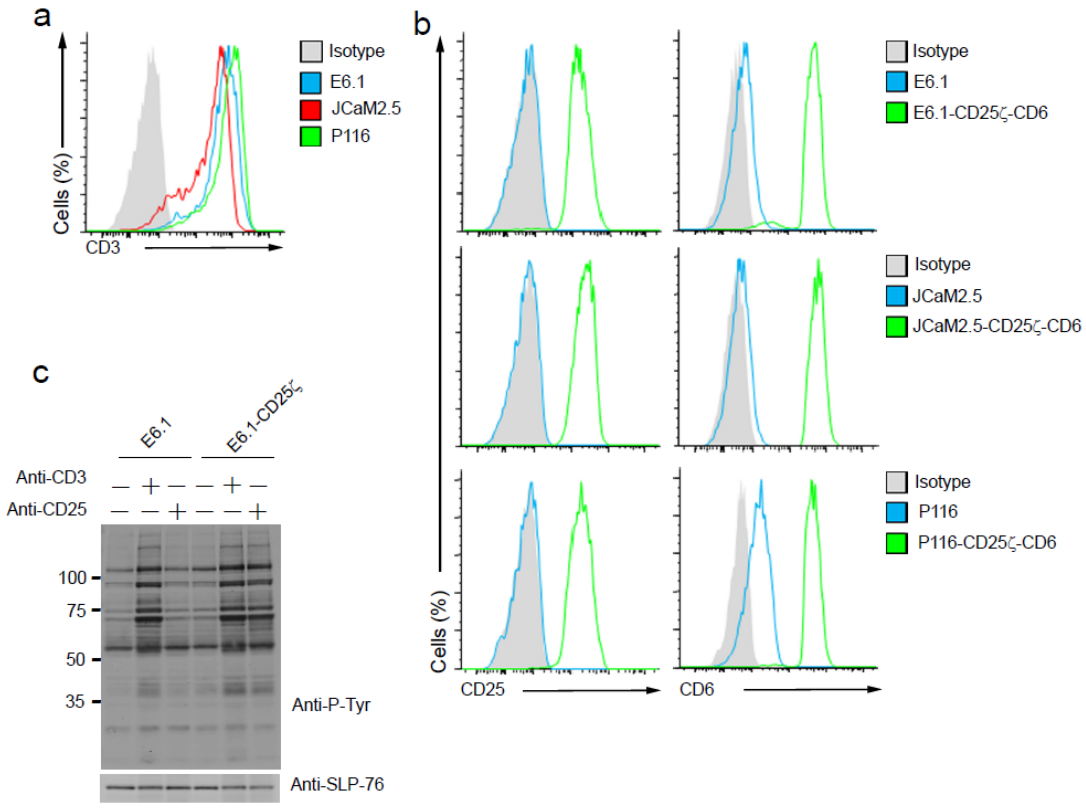
Supplementary Figure 3. Normal development and function of T cells isolated from knock-in mice homozygous for the *LAT*^{OST} allele. (**a**) Flow cytometry analysis of thymus and spleen. Wild-type (WT) and *Lat*^{OST} thymocytes were analyzed for expression of CD4 and CD8 (left) and TCRαβ and TCRγδ (right). Numbers adjacent to outlined areas indicate percent double-positive cells (top right), CD4^{+} single-positive cells (top left), CD8^{+} single-positive cells (bottom right) and double negative CD4^{-}CD8^{-} cells (bottom left). Wild-type and *Lat*^{OST} splenocytes were analyzed for expression of CD5. Numbers adjacent to outlined areas indicate percent B cells (top left) and T cells (bottom right). Expression of CD4 and CD8 and of TCRαβ and TCRγδ by T cells (identified as B220^{-}CD5^{+} cells). Numbers adjacent to outlined areas indicate percent CD4^{+} and CD8^{+} cells (middle) and TCRαβ and TCRγδ cells (right). Data are representative of at least three experiments with two mice per genotype. (**b**) Cellularity of thymus, pooled axillary, brachial, inguinal and mesenteric lymph nodes (LN) and spleen of wild-type and *Lat*^{OST} mice. Data are expressed as mean value ± SEM (n=6). (**c**) CFSE dilution by wild-type, and *Lat*^{OST} CD4^{+} T cells activated for 72 h with plate-bound anti-CD3 (3 μg/ml) in the presence or absence (key) of soluble anti-CD28 (1 μg/ml). Black lines, CFSE dilution at initiation of culture. (**d**) IL-2 in supernatants of wild-type, and *Lat*^{OST} CD4^{+} T cells activated for 72 h as in (**c**). (**e**) Changes in intracellular calcium in wild-type, and *Lat*^{OST} CD4^{+} T cells stimulated with biotinylated anti-CD3 and avidin. Arrow corresponds to the time of addition of avidin. Data are representative of at least two experiments. Wild-type and *Lat*^{OST} CD8^{+} T cells were analyzed in parallel and showed similar changes in intracellular calcium (data not shown).



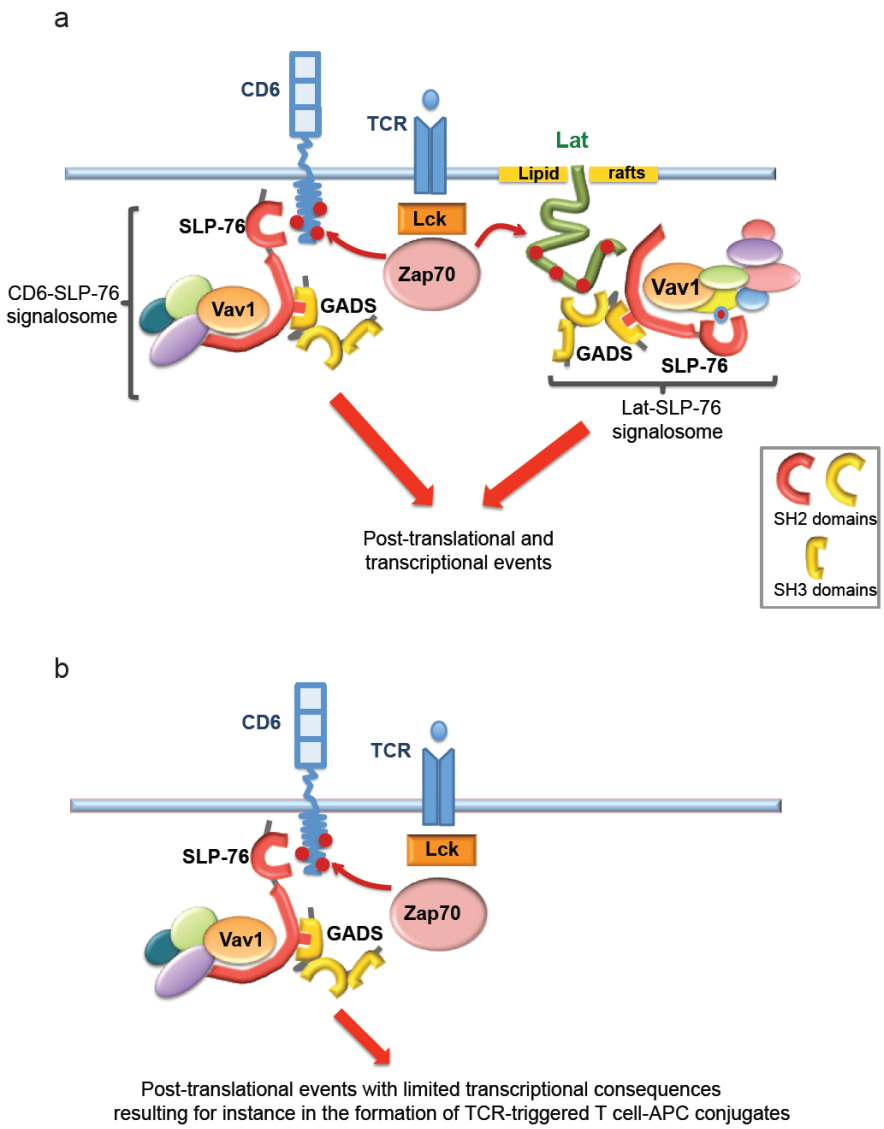
Supplementary Figure 4. Flow chart of quantitative mass spectrometry of primary CD4$^+$ T cells. (**a**) Schematic overview of affinity purification and mass-spectrometry (AP-MS) analysis of primary mouse CD4$^+$ T cells isolated from knock-in mice expressing endogenous proteins belonging to the TCR signaling pathway and tagged with a One-STrEP-tag (OST). (**b**) Representation of dynamic changes in the indicated THMS1 peptide LTLLSLAEER during pervanadate stimulation by label-free quantification of the precursor ion intensity.



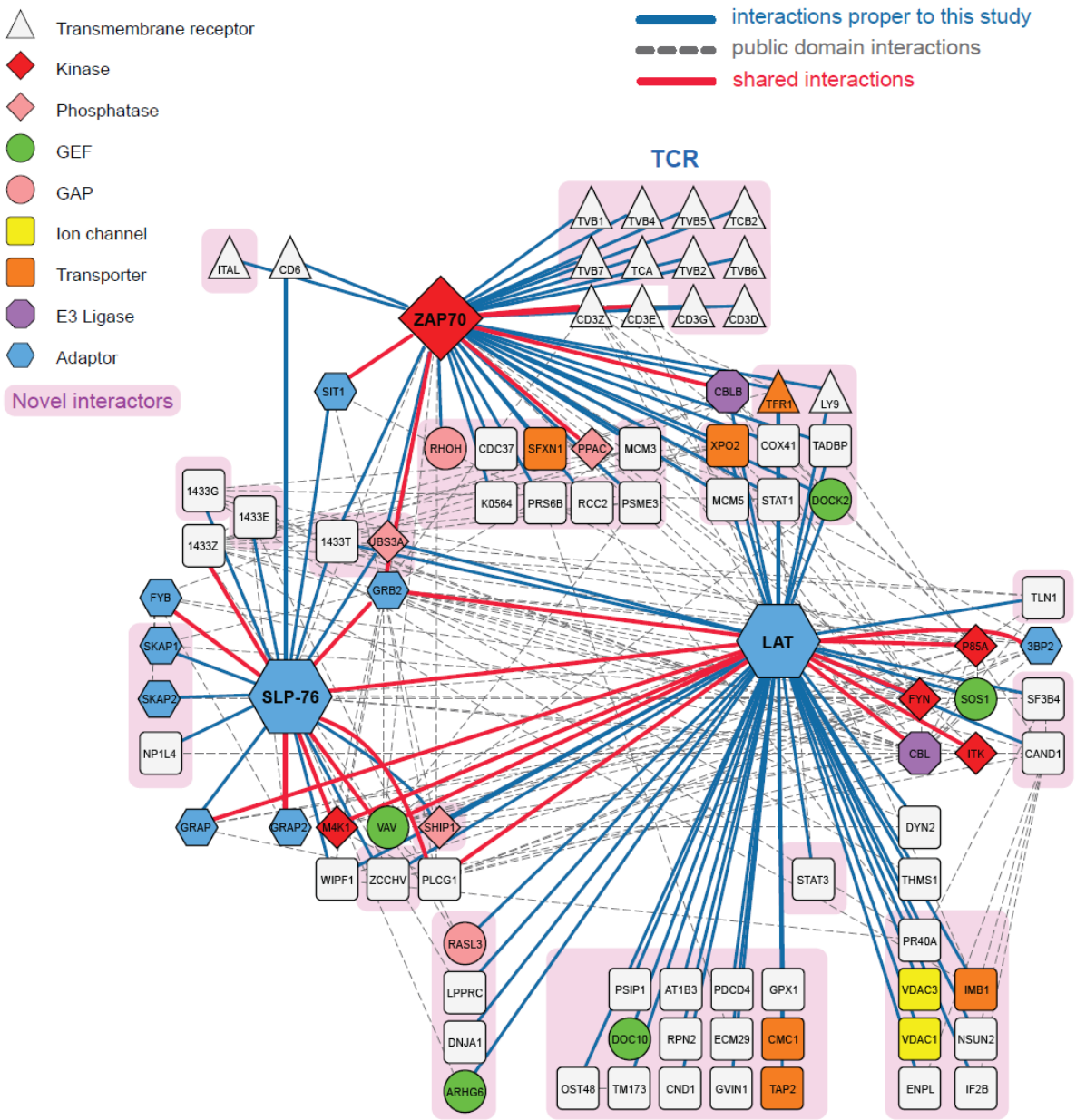
Supplementary Figure 5. The Zap70-Lat-SLP-76 network is enriched for proteins with domains involved in protein-protein and in protein-phospholipid interactions. (**a**) Protein domain classification[1] is showed in the right margin. The graph shows the distribution of the specified molecular domains among the proteins of the Zap70-Lat-SLP-76 network. Statistical validation of the enrichment for Src-homology 2 (SH2) and 3 (SH3) domains and Pleckstrin Homology (PH) domains is shown below the graph. (**b**) Representation of Protein-Protein Interactions (PPIs) identified in this study and in public PPI databases[2]. The numbers of interactions corresponding to each of the specified category (see key) are shown. The new interactions identified in this study are displayed in blue. Previously described interactions not identified in this study are categorized on the basis of their occurrence (green: one occurrence; purple: more than one occurrence). Shared interactions between the PINA database and this study are shown in red.



Supplementary Figure 6. CD25 and CD6 expression in Jurkat E6.1 T cells and their Zap70-deficient (P116) and Lat-deficient (JCaM2.5) variants prior to or after transfection with CD25ζ and human CD6 constructs. (**a**) Expression of the CD3ε chains on the specified cells (see key). An isotype control staining is also shown (control). (**b**) CD25 and CD6 expression on the specified cells. An isotype control staining is also shown (control). (**c**) E6.1 and E6.1 E6.1-CD25ζ-CD6 cells were left untreated (-) of stimulated (+) with anti-CD3 or anti-CD25 for 2 min at 37°C. Equivalent amounts of lysates were analyzed by immunoblot with antibody specific for phosphotyrosine (Anti-P-Tyr). An anti-SLP-76 was used as a loading control.



Supplementary Figure 7. Early TCR signals proceed via two distinct signal nucleation platforms involving the Lat molecule and the CD6 surface receptor. **(a)** Right part. Once bound to their antigenic ligand, TCR expressed on the surface of T cells recruit the protein tyrosine kinase Lck and Zap70 that in turn phosphorylates the tyrosine residues (red dots) found in Lat molecules. As a result, phosphorylated Lat molecules recruit the adaptors SLP-76-GADS and the assembly of a Lat-SLP-76 signalosome ensues. Note that SLP-76 constitutively associates with the GADS adaptor and uses the SH2 domain of GADS to dock onto phosphorylated Lat molecules. Left part. The CD6 surface receptor is recruited to the immunological synapse via interaction with CD166 on antigen-presenting cells. The docking of the SLP-76-GADS complex to phosphorylated CD6 occurred through the SLP-76 SH2 domain[3]. In contrast to CD6, Lat is palmitoylated and associated with lipid rafts. Accordingly, the Lat- and CD6-based signalosomes may be located within distinct plasma membrane lipidic environments and contribute non-redundant signals. (**b**) In the absence of Lat, the TCR initiates the formation of an incomplete CD6-SLP-76 signalosome that is capable of inducing a number of post-translational events that have limited transcriptional consequences but result for instance in the formation of TCR-triggered T cell-APC conjugates.



Supplementary Figure 8. Combined Cytoscape map of the Zap70-Lat-SLP-76 network based on public human and mouse protein-protein interaction (PPI) data and on PPIs observed in this study. The overall organization of the map is as shown in Fig. 3 and protein-protein interactions were visualized using Cytoscape 2.8.3. Colors and shapes of nodes indicate the type of identified proteins according to their domains and putative functions (key shown in the top right hand corner). Edge line colors define the PPI that are proper to the present study, the PPI that are found in public database, and the PPI that are found in both the present study and public database (shared interactions). Pink boxes highlight the subsets of proteins that interact with Zap70, Lat or SLP-76 and have not been previously identified in public databases. Each of the proteins is denoted by its Uniprot symbol (see **Supplementary Table 1**) except LCP2 that is called SLP-76.

Supplementary Table 1. List of the proteins associated with Zap70, Lat and SLP-76 in resting and pervanadate-activated $CD4^+$ T cells (Figure 3) and with SLP-76 in anti-CD3 and anti-CD4 activated $CD4^+$ T cells (Figure 2d) and that were identified in the present study by affinity purification and mass-spectrometry. Each of the proteins is denoted by its Uniprot symbol (http://www.uniprot.org) except LCP2 that is called SLP-76.

Supplementary Table 2. List of the protein-protein interactions identified in the present study and the PINA public PPI database. Direct interactions with the bait and inter-prey interactions are listed. When available, the mouse or human interacting proteins are characterized by their Uniprot AC number. References (PubMed; http://www.ncbi.nlm.nih.gov/pubmed) and methods of analysis are provided for each specified interaction.

Supplementary Table 3. List of genes differentially expressed in Lat^+CD4^+ T cells activated for 4 hours with anti-CD3 and anti-CD28 antibodies versus Lat^+CD4^+ T cells kept for 4 hours without stimuli and in Lat^-CD4^+ T cells activated for 4 hours with anti-CD3 and anti-CD28 antibodies versus Lat^-CD4^+ T cells kept for 4 hours without stimuli. Data were normalized and differentially expressed genes were identified based on adj.p > 0.05 and logFC >1 or <-1.

References

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